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ORIGINAL ARTICLE

Clustering sperm: A statistical approach to identify sperm morph numbers in the *Drosophila obscura* species group

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Abstract

The *Drosophila obscura* species group is sperm heteromorphic. Sperm heteromorphism is the production of multiple sperm types, or 'morphs', within a single male. In the case of the *obscura* species, males produce two or three sperm morphs of different sizes, simultaneously, within the same testis, and throughout their lifetime. A long sperm morph, the 'eusperm' is fertile, whereas shorter sperm morphs, the 'parasperm', are non-fertilising and protect the eusperm within the female reproductive tract. Many studies over the past 55 years have measured the sperm length of eusperm and parasperm in *obscura* species. One recent study found that *D. pseudoobscura* produces three sperm morphs, two parasperm and one eusperm, in contrast to the two morphs described in older studies of this species. Thus, the number of morphs made by some species in this clade is still in dispute. Here, we review sperm length data from previous studies and re-measure sperm of eight species including *D. pseudoobscura*. We used two statistical cluster analysis approaches to identify sperm morphs based on sperm and nucleus length, to test whether multiple parasperm morphs are present in more species than previously identified. We confirmed the presence of two parasperm morphs in *D. pseudoobscura*, and found that two closely related species, *D. persimilis* and *D. miranda*, also produce two parasperm morphs. *D. affinis*, *D. azteca*, *D. bifasciata*, *D. guanche* and *D. subobscura* produce a single parasperm morph, indicating that the presence of two parasperm is a derived feature in the *pseudoobscura* species subgroup.

KEYWORDS
fertilisation, *Drosophila pseudoobscura*, eusperm, parasperm, sperm heteromorphism, statistical clustering

INTRODUCTION

Sperm heteromorphism

Sperm heteromorphism is the production of multiple sperm morphs by an individual male. Morphs may be produced simultaneously or sequentially, and within the same testis or in separate testes. Morphs are produced consistently between males and are not the result of developmental aberration. Sperm morphs are distinct in morphology and function. For example, they may vary in size and/or DNA content. One or more morphs is non-fertilising, having some other function in fertility and reproduction (Bernasconi & Hellriegel, 2005, Snook et al., 1994, Snook & Karr, 1998, Swallow & Wilkinson, 2002).

The *obscura* species group (Figure 1) is the only *Drosophila* known to produce multiple sperm morphs, producing two or more morphs of differing sizes, a form of sperm heteromorphism sometimes termed 'polymegaly' (Beatty & Sidhu, 1970). In these species, a longer morph – the 'eusperm' – is fertile, while one or more shorter

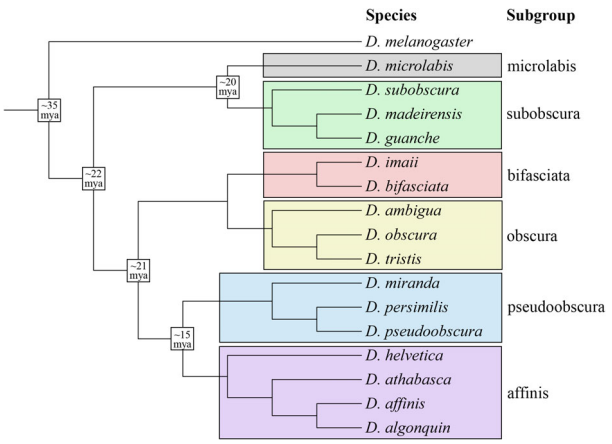

FIGURE 1 Phylogeny of the obscura species group, based on the *NADH2* gene. Divergence times from Gao et al., 2007. *D. azteca* (*affinis* species subgroup) is not included as no genome annotation is available.

morphs – the 'parasperm' – are non-fertilising. Shorter parasperm protect the longer eusperm from female-mediated spermicide present in the female reproductive tract (Alpern et al., 2019; Holman & Snook, 2008).

The number of morph classes and their functions has been debated since the phenomenon was first described in the *obscura* species by Beatty and Sidhu (1970). This research has spanned many decades and multiple research groups, and the number of morphs identified in each species has not always been conclusive. In this work, we have synthesised previous research findings describing heteromorphic sperm in the *obscura* species (Table 1) and provide a new statistical analysis of sperm length data in eight *obscura* group species, to clarify the number of sperm morphs present in these species.

Inter- and intra-specific variation in sperm length

Joly and Lachaise (1994) analysed sperm length variance within and between males, and between strains of *D. affinis*, finding significant differences in the length of long sperm between individuals and populations, and in the length of short sperm between individuals within populations. They also compared sperm length between closely related species (within species subgroups), finding greater interspecific variation in eusperm length compared with parasperm, based on the coefficient of variation.

Broadly, *Drosophila* sperm length is highly correlated with the length of the female sperm storage organs, the spermathecae (Pitnick et al., 1999). In *obscura* species specifically neither eusperm length nor parasperm length was correlated to spermathecal area. However, eusperm length, but not parasperm length, is correlated with the length of the female ventral receptacle, suggesting the morphs have evolved differing functions (Holman et al., 2008). Quantitative genetics experiments have shown that parasperm have higher evolvability than eusperm, suggesting it is not constrained by the same selection pressures as eusperm to maintain fertilisation function (Moore et al., 2013).

Functions of parasperm in the *obscura* species group

Both eusperm and parasperm contain an equal amount of DNA; however, only eusperm, and never parasperm, are observed in the fertilised eggs of *D. pseudoobscura*, suggesting that only eusperm are capable of fertilisation (Snook et al., 1994). This analysis was later expanded to over 1500 eggs from *D. affinis*, *D. athabasca*, *D. miranda*, *D. persimilis* and *D. subobscura*, which were also found to contain only eusperm sperm (Snook & Karr, 1998). Snook and Karr (1998) suggested that there may be a barrier to parasperm entry into the egg via sperm–egg interactions, such as a lack of gamete surface membrane receptors or parasperm heads being too wide to enter the micropyle.

Instead of fertilisation, parasperm have a protective role. The female reproductive tract is a spermicidal environment, in which eusperm viability declines over time. When incubated with female reproductive tract tissue extract, increasing the proportion of parasperm in the ejaculate increases eusperm survival. Males which transfer a higher proportion of parasperm in the ejaculate also show higher eusperm viability both after 30 minutes and 2 hours in the female reproductive tract. This indicates that parasperm protect eusperm in the female reproductive tract (Alpern et al., 2019; Holman & Snook, 2008; Peckenpaugh et al., 2021).

Where multiple parasperm morphs are present, the medium parasperm 2 may also have a function in sperm competition. Alpern et al. (2019) found increased sperm competition increases the percentage of parasperm 2 in the ejaculate, while eusperm and parasperm 1 percentages decrease. They suggest that parasperm 2 may remove sperm of competing males already present in the female reproductive tract and spermathecae.

Sperm heteromorphism in the *obscura* species group

Eusperm and parasperm length data are available for twenty *obscura* group species (Table 1). For some, further morphometric data is available, including nucleus length and proportions of each morph in the ejaculate. Several of these species have been measured multiple times, with variations observed between studies that may reflect natural population variation or methodological differences, and these are discussed in more detail below.

Affinis – D. affinis, D. azteca, D. algonquin, D. athabasca, D. helvetica, D. tolteca and *D. narraganset*

The *affinis* subgroup was first investigated by Sanger and Miller (1973) then later by Joly et al. (1989), finding two classes in all seven species. The *affinis* subgroup has the greatest variability between species ranging from parasperm lengths of 100 μm in *D. helvetica* to

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TABLE 1 Summary of the number of sperm morphs identified, and total lengths and nucleus lengths of heteromorphic sperm in the *obscura* species group in published literature.

Subgroup[a]	Species	*n* Sperm Morphs	Total sperm length (μm)			Nucleus length (μm)			Reference(s)
			Short	Medium	Long	Short	Medium	Long	
affinis	*D. affinis*	2	120–130		500–520				Sanger and Miller (1973)[c]
		2	60–190		390–540			50–100	Chang and Miller (1981)
		2	112		424				Joly et al. (1989), Joly and Lachaise (1994)
		2	147.7		569.0	25.1		92.8	Bircher and Hauschteck-Jungen (1997)
		2	130		506	20		91	Snook (1997)
	D. algonquin	2	160–190		960–1110				Sanger and Miller (1973)[c]
		2	90–170		820–1100			180–330	Chang and Miller (1981)
		2	150		894	18		251	Snook (1997)
	D. athabasca	2	120–140		1290–2060				Sanger and Miller (1973)[c]
		2	90–190		1180–1940			240–510	Chang and Miller (1981)
		2	118		1527	16		449	Snook (1997)
	D. azteca	2	180–230		1820–2080				Sanger and Miller (1973)[c]
		2	130–240		1780–2310			330–540	Chang and Miller (1981)
		2	143		925				Joly et al. (1989), Joly and Lachaise (1994)
		3	209.2	389.4	1875.6	15.3	53.3	494.8	Bircher and Hauschteck-Jungen (1997)
		2	174		1433	15		351	Snook (1997)
	D. helvetica	2	100		223				Joly et al. (1989), Joly and Lachaise (1994)
	D. narragansett	2	200		800–930				Sanger and Miller (1973)[c]
		2	160–230		870–970			40–90	Chang and Miller (1981)
	D. tolteca	2	130–140		1680–2000				Sanger and Miller (1973)
		2	90–160		1720–2330			330–530	Chang and Miller (1981)
		2	98		303	15		56	Holman et al. (2008)
bifasciata/ imaii[b]	*D. bifasciata*	2	83		228				Joly et al. (1989), Joly and Lachaise (1994)
	D. imaii	2	69		208	19		65	Holman et al. (2008)
obscura	*D. ambigua*	3	46	113	257				Beatty and Sidhu (1970)
	D. ambigua (British Colombia)	2	102		313	17		60	Snook (1997)
	D. ambigua (UK)	2	86		310	14		58	Snook (1997)
	D. obscura	3	93	180	274				Beatty and Sidhu (1970)
		2	76		139				Bressac et al. (1991), Joly et al. (1989), Joly and Lachaise (1994)
		2	96		230	23		51	Snook (1997)

(Continues)

TABLE 1 (Continued)

Subgroup[a]	Species	*n* Sperm Morphs	Total sperm length (μm)			Nucleus length (μm)			Reference(s)
			Short	Medium	Long	Short	Medium	Long	
	D. tristis	2	112		235				Joly et al. (1989), Joly and Lachaise (1994)
pseudoobscura	*D. miranda*	2	87		309	13		61	Snook (1997)
	D. persimilis	3	67	109	295				Beatty and Sidhu (1970)
		2	67		244				Joly et al. (1989), Joly and Lachaise (1994)
		2	77		324	13		70	Snook (1997)
	D. pseudoobscura	3	63	118	331				Beatty and Sidhu (1970)
		2	56		263				Bressac et al. (1991), Joly et al. (1989), Joly and Lachaise (1994)
		2	56.6		292.9	14.2		56.6	Snook et al. (1994)
		3	54.49	101.63	304.77				Alpern et al. (2019)
	D. pseudoobscura bogotana	2	108		404	19		78	Snook and Karr (1998)
microlabis	*D. kitumensis*	2	87		248				Joly et al. (1989), Joly and Lachaise (1994)
	D. microlabis	2	68		196				Bressac et al. (1991), Joly et al. (1989), Joly and Lachaise (1994)
subobscura	*D. guanche*	2	131		273				Joly et al. (1989), Joly and Lachaise (1994)
	D. madeirensis	2	137		218				Joly et al. (1989), Joly and Lachaise (1994)
	D. subobscura	2–4	74	122	232 (430[d])				Beatty and Sidhu (1970)
		2	85		199				Bressac et al. (1991), Joly et al. (1989), Joly and Lachaise (1994)
		2	149.6		257.8	32.6		49.6	Bircher and Hauschteck-Jungen (1997)
	D. subobscura (Europe)	2	197		327	15		55	Snook (1997)
		2	154		303	32		52	Snook and Karr (1998)
	D. subobscura (North America)	2	256		488	37		88	Snook (1997)
		2	120		224	18		46	Snook and Karr (1998)

Note: The gray shades indicate missing values. either because no medium sperm were found, or because nucleus length wasn't measured (or both for column 8).

[a]Species subgroups according to O'Grady (1999), Barrio and Ayala (1997), Gao et al. (2007).

[b]Older literature grouped *D. bifasciata* and *D. imaii* with the *obscura* species, however some more recent studies have found the *bifasciata/imaii* and *obscura* subgroups to be paraphyletic.

[c]Sanger and Miller (1973) measured multiple populations for each species, however data is shown as a range as all populations were from similar geographical distributions.

[d]430 μm sperm in *D. subobscura* observed once.

240 μm in *D. azteca*, and eusperm lengths of 220 μm up to 2310 μm, again in *D. helvetica* and *D. azteca*, respectively (Chang & Miller, 1981; Joly et al., 1989).

A later study by Bircher and Hauschteck-Jungen (1997) used cluster analysis (method not specified) of sperm length to identify the number of morph classes in both *D. affinis* and *D. azteca*. They found two morphs in *D. affinis*, but three in *D. azteca*, although they note that the "medium" morph in *D. azteca* was observed in only two of the five males.

Obscura – *D. obscura*, *D. ambigua* and *D. tristis*

D. obscura and *D. ambigua* were both included in Beatty & Sidhu's, 1970 study (Beatty & Sidhu, 1970). Three morph classes were identified in *D. obscura*, 93, 180 and 274 μm in length, and *D. ambigua*, 46, 113 and 257 μm in length. Subsequent studies have not identified a 'medium' class, finding only two morph classes in both species. *D. tristis* was also found to have two morph classes, 112 and 235 μm in length (Joly et al., 1989).

D. obscura parasperm has been measured to be between 76 and 96 μm, consistent with earlier studies (Bressac et al., 1991; Joly et al., 1989; Joly & Lachaise, 1994). However, eusperm length varies considerably between these studies, 139 μm in Joly et al. (1989), and 230 μm in Snook (1997), closer to that found by Beatty and Sidhu (1970). This variation in eusperm length may be due to naturally occurring population variation, as has been observed in other species (Joly & Lachaise, 1994; Snook, 1997; Snook & Karr, 1998).

Snook (1997) measured sperm and nucleus length in two populations of *D. ambigua*, noting a small difference in lengths between UK and Canadian populations. In both cases, two morphs were identified, 86–102 μm and 310–313 μm.

Pseudoobscura – *D. pseudoobscura*, *D. persimilis* and *D. miranda*

Arguably the most studied species within the *obscura* species group, *D. pseudoobscura* was originally described as having three sperm morphs: a short 63 μm morph, a medium 118 μm morph and a long 331 μm morph (Beatty & Burgoyne, 1971; Beatty & Sidhu, 1970). *D. persimilis* was also described as having three morphs, 67 μm, 109 μm and 295 μm in length, although the shorter morphs were considered 'indistinctly separate' (Beatty & Burgoyne, 1971, Beatty & Sidhu, 1970). *D. pseudoobscura* and *D. persimilis* were revisited in a larger study by Joly et al. (1989) and Joly and Lachaise (1994), which found a bimodal distribution of morphs in both species. A small number of 'extra-long' sperm were observed in *D. pseudoobscura*, although this was not further discussed (Joly & Lachaise, 1994). Snook et al. (1994) found evidence for only two sperm morphs in *D. pseudoobscura*, although they noted that there was a possibility for the presence of two 'short' parasperm size classes, which would be difficult to distinguish. Sperm head (nuclear) length was also characterised, finding distinct head lengths for the two size classes identified (parasperm = 14.2 μm, eusperm = 56.6 μm).

D. pseudoobscura was revisited again by Alpern et al. (2019), who found further evidence in support of earlier observations by Beatty and Sidhu (1970) that there are three sperm morphs produced in this species. They identified two distinct parasperm morphs, for which the lengths were non-overlapping: 55 μm parasperm 1 and 102 μm parasperm 2. Using a statistical approach, they confirmed that the three groups were significantly different from each other, based on total sperm length (ANOVA with Tukey's post-hoc test). Morphological differences between the parasperm morphs were observed: a 'string-like structure' associated with the tails of parasperm 2 and eusperm, which appears to be required for the spiral conformation of the tail of these two morphs but was not observed in parasperm 1.

Subobscura – *D. subobscura*, *D. guanche* and *D. madeirensis*

D. subobscura was initially suggested to have up to four morphs by Beatty and Sidhu (1970), finding 74 μm short, 122 μm medium, 232 μm long and 430 μm extra-long sperm, although only one 430 μm was observed in that study. *D. subobscura* has been re-measured more than any other species, across multiple populations and geographic distributions, and a substantial variation in both parasperm and eusperm lengths is apparent. Parasperm length varies between 85 and 256 μm, with eusperm length between 199 and 488 μm (Beatty & Burgoyne, 1971; Bircher & Hauschteck-Jungen, 1997; Bressac et al., 1991; Joly et al., 1989; Joly & Lachaise, 1994; Snook, 1997; Snook & Karr, 1998). In cluster analysis of *D. subobscura* sperm length, Bircher and Hauschteck-Jungen (1997) found two morph classes, as have all other studies subsequent to Beatty and Sidhu (1970).

Relative proportions of sperm morphs

The proportion of eusperm to parasperm within the ejaculate can be measured by various methods: counts of individualising spermatid cysts, sperm extracted from seminal vesicle ('produced'), or sperm extracted from female reproductive tract ('transferred').

Published data on parasperm proportion in ejaculate is shown in Table 2. Despite different methodologies and sample populations, proportion remains relatively consistent between studies. Proportions can change as the male ages; one-day-old *D. subobscura* males have almost no eusperm in the seminal vesicle, whereas seminal vesicles of four-day-old males contain both morphs (Bircher et al., 1995). This suggests that eusperm require more time to develop than parasperm. There may also be an effect of rearing stocks in the lab. In comparison with wild-caught populations, laboratory-reared *D. pseudoobscura* transfer an increased proportion of eusperm (Snook & Markow, 2002).

Between species, the proportion of parasperm in the ejaculate correlates with eusperm length. Parasperm comprise a greater proportion of the ejaculate in species with longer eusperm (Holman et al., 2008).

Statistical methods for analysis of sperm morph classes

While most studies have used a visual approach to assigning morph classes, statistical methods to determine the number of sperm morphs and their significance have been used in some previous studies of sperm heteromorphism. Snook (1995) used an approach of testing sperm length distributions for normality, then describing the shape of the distribution by kurtosis. Kurtosis indicated most species in the *obscura* species group produce two morphs, but for *D. pseudoobscura*, *D. miranda* and a European population of *D. ambigua*, kurtosis indicated two parasperm morphs – short and medium, although these were not discrete. Alpern et al. (2019) used ANOVA to test for significance in the difference of sperm lengths between putative morphs in *D. pseudoobscura*. Short, medium and long morph total sperm length was significant by Tukey's post-hoc. They also found a significant difference in nucleus length between parasperm 1 and 2.

TABLE 2 Proportion of parasperm in the ejaculate of obscura group species, by species subgroup. Method of sperm extraction is specified. Produced = sperm dissected from seminal vesicle

Subgroup	Species	Parasperm %	Method	References
affinis	*D. affinis*	70	Produced	Joly and Lachaise (1994)
		75	Produced	Snook and Markow (2001)
		72	Transferred	
	D. athabasca	94	Transferred	Holman et al. (2008)
	D. azteca	88	Produced	Joly and Lachaise (1994)
	D. helvetica	42	Produced	
	D. tolteca	69	Transferred	Holman et al. (2008)
bifasciata	*D. bifasciata*	30	Produced	Joly and Lachaise (1994)
	D. imaii	42	Transferred	Holman et al. (2008)
microlabis	*D. kitumensis*	70	Produced	Joly and Lachaise (1994)
	D. microlabis	63	Produced	
obscura	*D. ambigua*	73	Transferred	Holman et al. (2008)
	D. obscura	41	Produced	Joly and Lachaise (1994)
	D. tristis	67	Produced	
pseudoobscura	*D. miranda*	56	Transferred	Holman et al. (2008)
	D. persimilis	49	Produced	Joly and Lachaise (1994)
		51	Produced	Snook and Markow (2001)
		50	Transferred	
	D. pseudoobscura	45	Produced	Joly and Lachaise (1994)
		44	Produced	Snook and Markow (2001)
		44	Produced	Snook et al. (1994)
		47	Transferred	Alpern et al. (2019)
		48	Transferred	Snook and Markow (2001)
subobscura	*D. guanche*	50	Produced	Joly and Lachaise (1994)
	D. madeirensis	64	Produced	
	D. subobscura	49	Produced	
		65	Spermatid cysts	Bircher et al. (1995)
		66.3	Transferred	Bressac and Hauschteck-Jungen (1996)

Note: Transferred = sperm dissected from female reproductive tract. Spermatid cyst = individualising sperm cysts dissected from testis.

In this study, we use a cluster analysis approach to group sperm into morph classes based on total length and nucleus length. We used two mathematically dissimilar clustering methods, hierarchical cluster analysis and Gaussian mixture modelling, and compare the results of both models.

Aim of study

The aim of this study is to reanalyse sperm length and the number of sperm morph classes in eight species of the *obscura* species group: *D. affinis*, *D. azteca*, *D. bifasciata*, *D. guanche*, *D. miranda*, *D. persimilis*, *D. pseudoobscura* and *D. subobscura*. All eight have previously been analysed; however, recent evidence of multiple parasperm morphs in *D. pseudoobscura* gives reason to reassess the number of morphs present in other species, particularly those to which it is most closely related – *D. persimilis* and *D. miranda*. We use a new statistical approach to identify morphs, based on cluster analysis of sperm morphological data. We also discuss whether there is evidence of multiple eusperm morphs in *obscura* group species.

METHODS

Fly stocks

D. pseudoobscura SLoB3 stocks were gifted by T. Price (University of Liverpool, UK). *D. miranda* stocks were gifted by D. Bachtrog (UC Berkeley, USA). All other stocks were obtained from the National Drosophila Species Stock Center (Cornell University, USA) and Kyorin-Fly Stock Center (Kyorin University, Japan) (Table 3).

TABLE 3 *obscura* species group stocks used for sperm isolation, imaging, and measurements.

Species subgroup	Species	Stock centre	Line
affinis	*D. affinis*	NDSSC	14,012–0141.2
	D. azteca	NDSSC	14,012–0171.03
bifasciata	*D. bifasciata*	NDSSC	14,012–0181.02
pseudoobscura	*D. miranda*	Gift from D. Bachtrog	MSH22
	D. persimilis	Kyorin	K-S11
	D. pseudoobscura	Gift from T. Price	SLoB3 (Show Low, Arizona)
subobscura	*D. guanche*	NDSSC	14,012–0141.2
	D. subobscura	NDSSC	14,011–0131.16

Sperm dissection and fixation

Testes were dissected into a drop of testis buffer (183 mM KCl, 47 mM NaCL, 10 mM Tris HCl, pH 6.8) with seminal vesicles attached. Seminal vesicles were separated from the testes and then transferred to a fresh drop of testis buffer. To remove sperm from the seminal vesicles, the seminal vesicles were held with a pair of forceps at one end, and the sperm were squeezed out by running a fine tungsten needle along the length of the seminal vesicle. Empty seminal vesicles were discarded. Sperm was separated in testis buffer by gently mixing it with a fine tungsten needle; 20 μL of sperm in testis buffer was transferred to a 1.5 mL tube. The mixture was gently pipetted 5X and then incubated at room temperature for 5 min to allow further dissociation. To fix, 20 μL 4% paraformaldehyde in PBS + 0.1% Tween 20 was added to the sperm/testis buffer mixture and incubated at room temperature for 5 min. Next, 40 μL mounting medium (80% glycerol, 2.5% n-propyl gallate, 1 μg/mL Hoechst 33258) was added to the fixed sperm and mixed by pipetting 5X. Then, 30 μL of the fixed sperm/mounting medium mixture was transferred to a microscope slide and a coverslip was added.

A minimum of four males and 200 sperm were counted from each species (see S1 ***for data).

Imaging and image analysis

Slides were imaged using the Olympus BX50 (Olympus Europa, Germany) with Hamamatsu ORCA-05G camera attachment and HCImage software (v.2,2,6,4 Hamamatsu Corp. 2011). Each sperm was imaged by phase contrast and fluorescence (405 nm) for whole-sperm and nucleus, respectively. Imaging was performed in transects across the slide to ensure unbiased sampling of the sperm population, except for *D. azteca*, in which all eusperm on the slide were imaged. Measurements were performed with the ImageJ Measurement tool (ImageJ 1.54f, NIH). Nucleus measurements were taken from the base of the nucleus to the tip of the sperm head.

Statistical analysis

All statistical analysis was performed with R Studio v.4.2.1 (R Core Team, 2022) (see S2 for R scripts, S3 for model outputs). Cluster analysis was performed using the total sperm length and nucleus length measurements, both measured in microns. The data was not scaled prior to analysis. Two clustering approaches were used, and results were compared between the two models. *D. pseudoobscura* data was used to establish the parameters of the two models, as recently published data have shown strong evidence of three sperm morphs in this species (Alpern et al., 2019).

Hierarchical cluster analysis (HCA) was performed with the 'HCA' function of the 'stats' package (R Core Team, 2022). The optimum number of clusters was first determined by gap statistic modelling (method = first maximum, bootstrap = 1000) (Tibshirani et al., 2001) using the 'cluster' package (Maechler et al., 2022). Total sperm length and nucleus length data were transformed into a distance matrix using the Euclidean distance. Hierarchical clustering was performed using the Ward distance. Clustering was assigned using the 'cutree' function, with the number of clusters specified by the gap statistic. The distribution of total sperm length within clusters assigned by HCA was tested for normality by Shapiro–Wilk (S4). Gaussian mixture modelling (GMM) was performed with the 'mclust' package (Scrucca et al., 2016). GMM estimates the number of clusters and assigns datapoints to clusters by model fitting, so no other methods were required to estimate the number of clusters prior to running the model. Sperm length distributions were tested for normality by Shapiro–Wilk test; if a cluster identified by modelling was a distinct sperm morph, it would be expected that the total sperm lengths within that cluster would form a unimodal normal distribution.

To determine the number of morphs in each species, both clustering models were considered. Where both models identified a cluster, this was considered to have greater statistical support than clusters identified by only a single model. Clusters were then assessed for distinctness: no or little overlap in the distribution of total and head length between clusters; and consistency, whether each cluster contained sperm from all males measured. Dissection of sperm from the seminal vesicle can result in sperm breaking, therefore we considered whether clusters were likely to comprise broken sperm of another cluster (such as nucleus length similar to that of eusperm but short or intermediate total length). Finally, we examined morphological characteristics of each cluster, such as tail coiling and head shape, which have previously been indicated to differ between morphs (Alpern et al., 2019). Tail coiling and head shape were subjective and, therefore, not included in HCA or GMM modelling.

All scatterplots and dendrograms were drawn with 'ggplot2' (Wickham, 2016) and 'ggdendro' (De Vries & Ripley, 2022) packages.

The proportion of parasperm within the ejaculate was calculated as:

$$\frac{n\,\text{Parasperm}}{n\,\text{Total Sperm}}$$

The proportion of parasperm length within the total sperm length produced was calculated as:

$$\frac{\sum \text{Parasperm Length}}{\sum \text{Parasperm Length} + \sum \text{Eusperm Length}}$$

RESULTS

Summary of results

Data for each species was plotted on a scatterplot of total sperm length against nucleus length (Figure 2). As has previously been observed, more than one sperm morph was present in all eight species studied. There was substantial variation between species in total sperm and nucleus lengths. The two cluster analysis methods produced different results in several species (Table 4). Gaussian mixture modelling often identified a greater number of clusters than HCA, suggesting it is a more sensitive method and/or is more prone to false positives. The proportion of total sperm length consisting of parasperm was variable between species, from 0.08 to 0.42, with a mean of 0.25 (S5).

In addition to length, there are other subjective morphological differences between eusperm and parasperm morphs (Figure 3). Eusperm nuclei are long and taper to a thin point at the tip of the sperm, whereas parasperm nuclei appear more rounded at the tip and taper towards the tail of the sperm. When imaged together, there is often a clear difference in the brightness of the DNA stain between the morphs, with parasperm nuclei being shorter, wider, and more compact, and thus appearing brighter. Parasperm nuclei often appeared hook- or 'C'-shaped compared with more linear eusperm nuclei, and parasperm tails were often more tightly coiled in appearance, while eusperm tails were wavier. The most likely number of sperm morphs, mean total sperm lengths, nucleus lengths, standard deviations, and proportion each morph contributes to the total sperm produced for each species are summarised in Table 5.

Affinis – *D. affinis* and *D. azteca*

D. affinis

D. affinis sperm was clustered into two clusters by HCA, with a further cluster identified by GMM of outliers likely to be predominantly broken eusperm. By HCA, neither parasperm nor eusperm lengths were normally distributed with outliers (parasperm $W = 0.500$, $p < 0.001$; eusperm $W = 0.859$, $p < 0.001$) but were normally distributed when the outliers were removed from the dataset (parasperm $W = 0.993$, $p = 0.251$; eusperm $W = 0.993$, $p = 0.372$). Mean parasperm length was 105.5 µm, mean eusperm length was 449.5 µm, and parasperm comprised 58% of the total sperm measured (Figure 3a,b).

D. azteca

Both HCA and GMM identified three clusters in *D. azteca*, one parasperm cluster, and two eusperm clusters. However, like *D. affinis*, it is likely that broken eusperm make up the shorter of the two eusperm clusters, as this cluster contains fewer individual sperm and shows large variation in total sperm length – between 1155 and 1743 µm. *D. azteca* has the longest eusperm of the *obscura* species group and therefore is more likely to break during dissection. Mean parasperm length was 232.4 µm. Mean eusperm length when broken sperm were removed from the dataset was 2068.1 µm (Figure 3c,d). Both parasperm and eusperm lengths, with broken eusperm removed from the data, were normally distributed (parasperm $W = 0.992$, $p = 0.301$; eusperm $W = 0.934$, $p = 0.106$).

Of the total sperm measured, 84% were parasperm, and 16% eusperm. However, due to the small numbers of eusperm produced by *D. azteca*, we measured all eusperm from each individual rather than a random transect of the slide, as was done for all other species. Therefore, the eusperm proportion of the total sperm measured is not representative of the eusperm proportion within the ejaculate for *D. azteca*.

D. bifasciata

Both HCA and GMM identified three clusters in *D. bifasciata*, one parasperm cluster and two eusperm clusters. Mean parasperm length was 83.7 µm and parasperm comprised 39% of the total sperm measured. Parasperm length was normally distributed ($W = 0.988$, $p = 0.356$).

Whereas in *D. affinis* and *D. azteca*, the third cluster was more obviously broken eusperm, this does not appear to be the case in *D. bifasciata*. *D. bifasciata* eusperm are much shorter than those of the *affinis* species subgroup, at a mean length of 258.8 µm, less likely to break during preparation. When the two HCA eusperm clusters were combined, the data were not normally distributed ($W = 0.97$, $p = 0.000932$). However, when separated, neither the HCA putative eusperm 1 ($W = 0.97$, $p = 0.021$) nor eusperm 2 ($W = 0.95$, $p = 0.014$) clusters were normally distributed. The question therefore is – does *D. bifasciata* produce two eusperm morphs, or is eusperm simply more variable in length than is observed in other species?

When clustered by HCA, representatives from both eusperm clusters were present in all individuals measured; by contrast, the GMM clustering did not identify sperm from both putative eusperm clusters in all males. Moreover, while both models identify two

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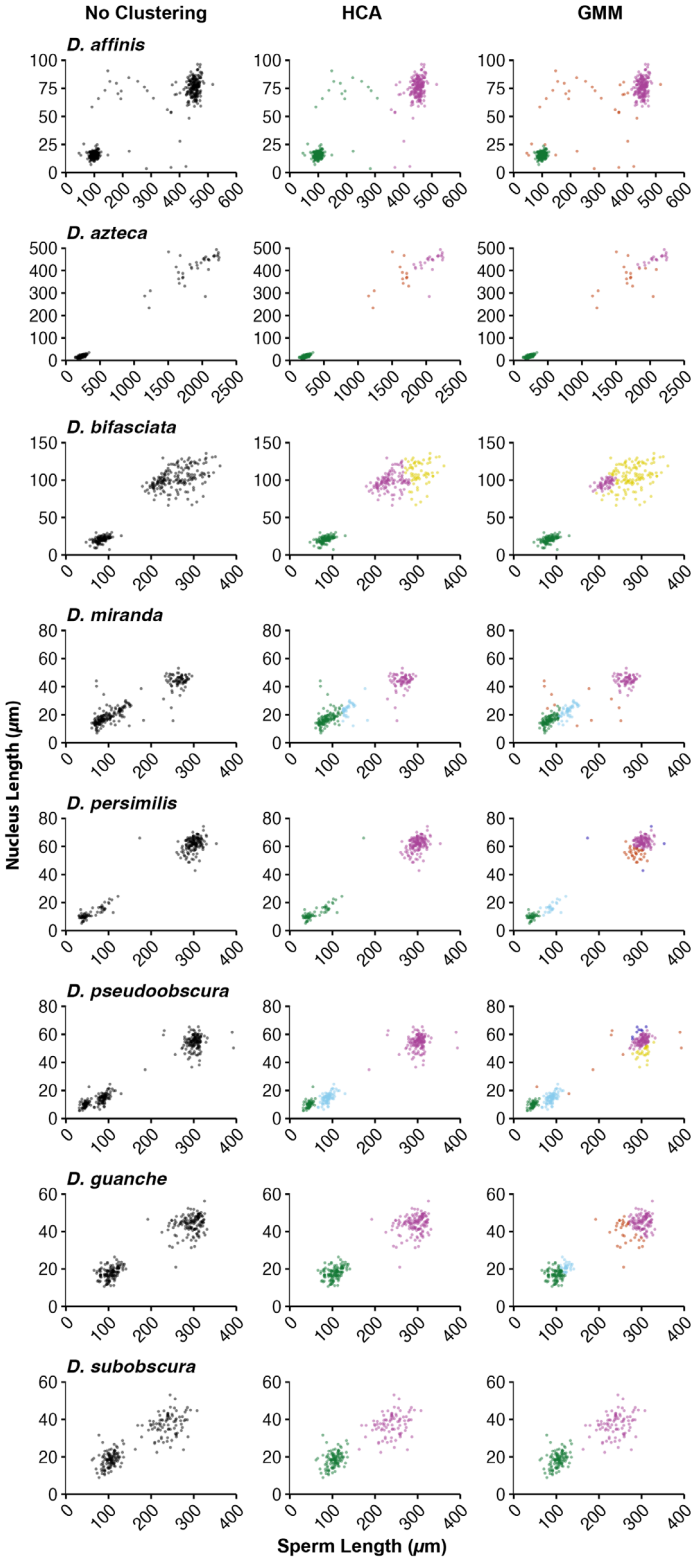

FIGURE 2 Scatterplots of total sperm length against nucleus length. For each species, data is shown with no clustering, clustering according to hierarchical cluster analysis (hclust) and Gaussian mixture modelling (mclust) of total sperm length and nucleus length data. Note different X and Y axis scales between species.

eusperm clusters, the cluster identity of individual datapoints is not consistent between the two models – 35% of eusperm morphs were placed in a different cluster in the GMM analysis compared with HCA. The mean lengths of the eusperm clusters, by HCA, were 234.4 and 304.4 μm, and by GMM were 215.4 and 277.7 μm. Mean nucleus length by HCA was 98.1 and 109.6 μm, and by GMM was 94.9 and

TABLE 4 Summary of number of clusters identified in sperm morphological data from *obscura* group species.

		n clusters	
Species subgroup	Species	Hierarchical cluster analysis	Gaussian mixture modelling
affinis	*D. affinis*	2	3
	D. azteca	3	3
bifasciata	*D. bifasciata*	3	3
pseudoobscura	*D. miranda*	3	4
	D. persimilis	2	5
	D. pseudoobscura	3	6
subobscura	*D. guanche*	2	4
	D. subobscura	2	2

Note: Two analysis methods were used, hierarchical cluster analysis and Gaussian mixture modelling.

103.9 μm. Nucleus length is notable for its similarity between the two clusters in both models. In subjective analysis of the images, other than length, there were no clear morphological differences between eusperm clusters (Figure 3e,f). There were considerable differences between individuals in the range of sperm lengths observed (S6). From this data, it is not possible to definitively conclude whether there are two distinct eusperm morphs; conservatively, we propose that the eusperm should be considered a single morph. Further morphological and functional analysis are required to resolve this.

Pseudoobscura – *D. miranda*, *D. persimilis* and *D. pseudoobscura*

D. pseudoobscura

HCA identified three clusters, two parasperm and one eusperm. Eusperm length was normally distributed after outliers were removed ($W = 0.982$, $p = 0.094$). Parasperm 1 length was normally distributed ($W = 0.974$, $p = 0.185$). Parasperm 2 length was normally distributed after outliers were removed ($W = 0.990$, $p = 0.724$). GMM also identified these clusters but additionally separated eusperm into multiple clusters. These clusters overlap and are therefore unlikely to be distinct morphs. Both parasperm 1 and 2 clusters were present in all individuals measured.

Consistent with the descriptions by Alpern et al. (2019), we found that morphology is distinct between the short and medium clusters. Short parasperm 1 have a wider head and less coiling in the tail, compared with medium parasperm 2 (Figure 3m–o). We therefore conclude that, as has previously been described, *D. pseudoobscura* produces two parasperm morphs and one eusperm morph. Mean length of parasperm 1 was 45.3 μm, parasperm 2 was 89.1 μm, and eusperm was 300.9 μm. Parasperm 1 comprised 22% of the total sperm measured, parasperm 2 30%, and eusperm 48%.

D. miranda

HCA identified three clusters in *D. miranda:* two parasperm clusters and one eusperm cluster. GMM identified an additional cluster, which is likely to be mostly broken eusperm. Mean eusperm length was 264 μm. Eusperm length was normally distributed ($W = 0.980$, $p = 0.280$).

Mean parasperm 1 length was 89.4 μm whereas mean parasperm 2 length was 137.1 μm. Neither parasperm 1 nor parasperm 2 length was normally distributed (parasperm 1 $W = 0.950$, $p < 0.001$; parasperm 2 $W = 0.804$, $p < 0.001$). Parasperm length was also not normally distributed when the clusters were combined ($W = 0.906$, $p < 0.001$), indicating that the clusters are separate, but not as distinct as *D. pseudoobscura*. The two parasperm clusters identified by GMM were present in all individuals from which sperm were measured, although by HCA clustering, none of the 27 sperm measured from male 2 were parasperm 2.

Morphological differences apparent in our images support the identification of two parasperm morphs. While the tails of both morphs were similarly coiled, the head and nucleus of parasperm 1 were often wider and hook-shaped, whereas the head and nucleus of parasperm 2 were often less curved, more like that of eusperm (Figure 3g–i).

We conclude that *D. miranda* produces three sperm morphs – parasperm 1, parasperm 2 and eusperm. Parasperm 1 comprised 53% of the total sperm measured, parasperm 2 15% and eusperm 32%.

D. persimilis

HCA and GMM produced considerably differing results for *D. persimilis*. HCA identified two clusters – eusperm and parasperm. The sperm length of the single parasperm cluster identified by HCA was not normally distributed ($W = 0.839$, $p < 0.001$) indicating that the parasperm cluster likely separates further into two subclusters, like that of *D. miranda* and *D. pseudoobscura*. GMM does identify these two parasperm subclusters but also identifies three eusperm clusters. As in *D. pseudoobscura*, these eusperm clusters overlap and are therefore unlikely to be distinct morphs. This conclusion is supported by the normal distribution of HCA eusperm lengths ($W = 0.991$, $p = 0.465$).

As with *D. pseudoobscura* and *D. miranda*, images of parasperm show two distinct morphologies. Shorter parasperm 1 showed less coiling of the tail compared with medium parasperm 2, although parasperm 1 was often observed to have coiled back on itself, forming a loop. Both parasperm 1 and 2 were often observed to have a hook-shaped nucleus, with parasperm 1 having a wider nucleus (Figure 3j–l).

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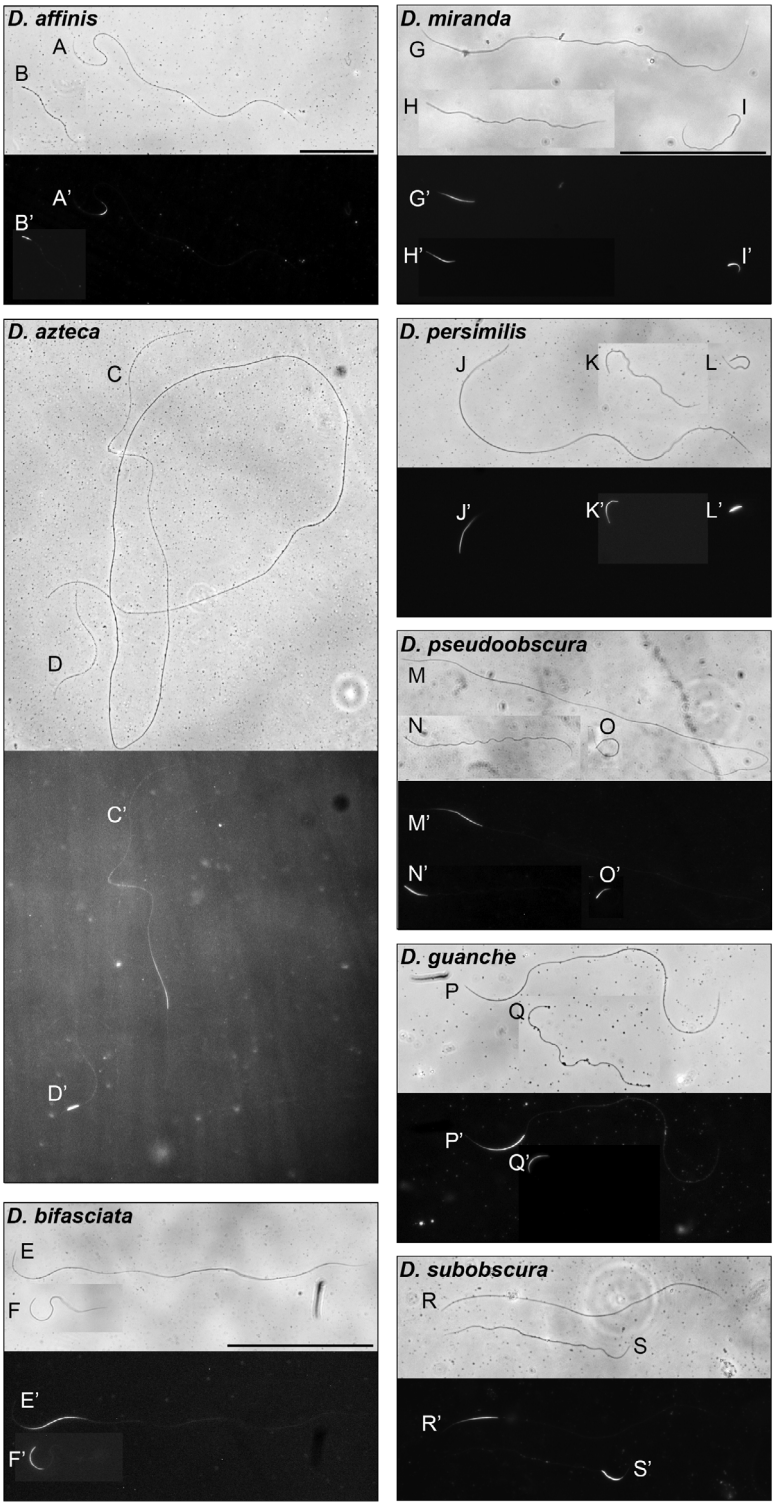

FIGURE 3 Mature spermatozoa from *Drosophila obscura* group species. A-S: Phase contrast imaging of whole sperm. A'-S': Fluorescence imaging of whole sperm showing nuclei stained with Hoechst 33258. A, C, E, G, J, M, P and R: Eusperm. B, D, F, Q and S: Parasperm. H, K and N: Parasperm 2, or medium sperm, from *D. miranda*, *D. persimilis*, and *D. pseudoobscura*. I, L and O: Parasperm 1, from *D. miranda*, *D. persimilis* and *D. pseudoobscura*. Note the long needle-shaped nuclei of eusperm compared with shorter parasperm nuclei, often hook or 'C' shaped. Phase contrast and fluorescence images brightness has been increased. Scale = 100 μm.

The two parasperm morphs were present in all individuals from which sperm were measured. Furthermore, mean morph sizes in *D. persimilis* were almost identical to those of its sister species, *D. pseudoobscura*. We conclude that, like *D. pseudoobscura* and *D. miranda*, *D. persimilis* produces three sperm morphs: short parasperm 1, medium parasperm 2, and long eusperm. Mean length of

TABLE 5 Means of sperm morphs based on HCA clustering.

Species	*n* sperm morphs	*n* males	*n* sperm measured	Mean sperm length μm (Std. dev.)			Mean nucleus length μm (Std. dev.)			Proportion of Total sperm measured		
				Short	Medium	Long	Short	Medium	Long	Short	Medium	Long
D. affinis	2	4	521	105.5 (29.1)		449.6 (20.6)	18.4 (12.7)		74.9 (10.7)	0.58		0.42
D. azteca[a]	2	5	241	232.4 (31.0)		2068.1 (134.6)	18.8 (4.2)		440.8 (39.7)	0.84		0.16
D. bifasciata	2[b]	6	308	83.7 (12.6)		258.8 (40.6)	20.7 (4.1)		102.1 (13.9)	0.39		0.61
D. miranda	3	5	239	86.4 (14.9)	137.1 (13.3)	264.0 (15.0)	17.2 (5.2)	23.9 (4.7)	43.9 (5.4)	0.53	0.15	0.32
D. persimilis	3[c]	5	216	42.6 (7.1)	89.1 (13.6)	299.1 (17.9)	9.9 (1.9)	16.8 (3.4)	61.6 (4.9)	0.20	0.11	0.69
D. pseudoobscura	3	5	294	45.3 (6.1)	89.1 (10.6)	300.9 (20.4)	10.2 (2.5)	14.8 (3.4)	54.3 (5.3)	0.22	0.30	0.48
D. guanche	2	5	276	104.9 (15.5)		293.2 (23.6)	17.9 (3.2)		34.7 (4.3)	0.48		0.52
D. subobscura	2	5	204	103.7 (14.5)		238.9 (28.7)	18.5 (4.0)		37.3 (6.0)	0.57		0.43

Note: The gray indicates no measurements as medium sperm are not present.

[a]*D. azteca* broken eusperm cluster was removed prior to calculating total sperm and nucleus length means, proportion of total sperm measured included broken eusperm in the 'long' cluster. Note that eusperm were not randomly sampled from *D. azteca*, therefore calculations for proportion of total sperm measured are not representative of proportion within the ejaculate.

[b]*D.bifasciata* HCA and GMM clustering identified two eusperm clusters, however clusters appeared to be resulting from individual variation. Two eusperm clusters were combined for mean total sperm length, nucleus length and proportion.

[c]*D. persimilis* HCA identified two clusters, GMM identified an additional parasperm cluster which is supported by morphological evidence.

parasperm 1 was 45.3 μm, parasperm 2 was 89.1 μm, and eusperm was 299.1 μm. Parasperm 1 comprised 20% of the total sperm measured, parasperm 2 11% and eusperm 69%.

Subobscura – *D. guanche* and *D. subobscura*

D. guanche

HCA and GMM gave different results for *D. guanche*. HCA identified two clusters, one parasperm and one eusperm. GMM identified four clusters, two parasperm and two eusperm. The two parasperm clusters were not as distinct as those of the *pseudoobscura* species subgroup. Examination of the images revealed no distinction in nuclear shape or tail coiling morphology between longer and shorter parasperm (Figure 3p,q). Sperm length of the single parasperm cluster identified by HCA was normally distributed ($W = 0.989$, $p = 0.358$), supporting the conclusion that there is a single parasperm morph in this species.

Of the eusperm clusters, the shorter appears, as with other species, to be outliers and broken eusperm. By HCA, eusperm length was not normally distributed ($W = 0.923$, $p > 0.001$). It was still not normally distributed after removal of outliers ($W = 0.977$, $p = 0.046$); visualisation of the data indicated eusperm length distribution was marginally skewed, but not bimodal (S3).

We conclude that *D. guanche* produces two morphs. Parasperm mean length was 104.9 μm, and eusperm mean length was 293.2 μm. Parasperm comprised 48% of the total sperm measured.

D. subobscura

Both HCA and GMM identified two clusters in *D. subobscura*. Both parasperm and eusperm lengths were normally distributed (parasperm $W = 0.989$, $p = 0.508$; eusperm $W = 0.991$, $p = 0.821$). Parasperm mean length was 103.7 μm; eusperm mean length was 238.9 μm. Parasperm comprised 57% of the total sperm measured.

DISCUSSION

The *obscura* group species produces multiple sperm morphs

The *D. obscura* species group is the only sperm heteromorphic *Drosophila* species. All *obscura* group species previously studied have been found to produce a long sperm morph – the eusperm, and a short sperm morph – the parasperm. In this study, we re-measured total sperm lengths and nucleus lengths of eight *obscura* group species: *D. affinis*, *D. azteca*, *D. bifasciata*, *D. miranda*, *D. persimilis*, *D. pseudoobscura*, *D. guanche* and *D. subobscura*. We also found that all eight produce at least two distinct sperm morphs, and some species – *D. miranda*, *D. persimilis* and *D. pseudoobscura* – produce three.

While sperm length and nuclear lengths were used for unbiased statistical modelling, we observed qualitative morphological differences between the eusperm and parasperm morphs. Parasperm were often more tightly coiled than eusperm and were often observed to have a hook-shaped nucleus. Some eusperm were also observed to have a bend in the nucleus, but this was less acute than that of parasperm. When imaged together, parasperm nuclei were often wider and brighter than eusperm nuclei. As both morphs contain the same DNA content (Pasini et al., 1996), this indicates more condensed DNA in parasperm nuclei, as might be required in a smaller volume nucleus. Similarly, in the pseudoobscura group, parasperm 1 nuclei were observed to be brighter and wider than parasperm 2 nuclei. These subjective, qualitative differences were not included in our statistical models. Development of robust methods for quantifying these differences would allow inclusion of more measurements in future clustering analyses.

Future work could also examine the differences in ultrastructure between eusperm and parasperm morphs, for example by electron microscopy (Pasini et al., 1996). This could also indicate developmental differences between morphs, such as in length, nuclear shaping and DNA condensation.

Sperm and nucleus lengths are consistent within species across studies

Mean sperm lengths for all species were consistent with previously published data. *D. bifasciata* eusperm mean length was slightly longer than previously published data (Joly et al., 1989), and *D. guanche* and *D. miranda* eusperm were shorter than in previously published data (Joly et al., 1989; Snook, 1997). However, all these species have been studied only once previously. For more commonly studied species, variability of mean sperm lengths is observed between studies and populations. It is likely therefore that this slight difference between data presented here and in previous studies reflects population and methodological variability.

We found no evidence of multiple parasperm morphs or 'giant' eusperm in *D. subobscura*, as was previously suggested in an early study by Beatty and Sidhu (1970).

D. miranda, *D. persimilis* and *D. pseudoobscura* produce three sperm morphs

D. pseudoobscura have recently been shown to have two parasperm morphs (Alpern et al., 2019). We wanted to assess whether its sister species, *D. persimilis*, and another closely related species, *D. miranda*, also produce two parasperm morphs, in addition to eusperm. We found that all three species of the *pseudoobscura* species subgroup produce three sperm morphs. The two parasperm morphs were distinct in length and morphology in all three species. It is also of note that *D. pseudoobscura* and *D. persimilis* have almost identical sperm and nucleus lengths across all three morphs, reflecting the relatively recent divergence between the two species (0.45–1.1 mya) (Korunes et al., 2021).

Previous studies have detected either two or three morphs in *D. pseudoobscura*. Differences in the number of morphs detected between studies might be due to genetic differences between strains. Length distributions of parasperm 1 and 2 may be more or less distinct, depending on the genetic background of the strain. Alternatively, there may be a methodological component; Joly et al. (1989), Joly and Lachaise (1994) and Snook et al. (1994) measured sperm dissected from seminal vesicle, finding two morphs, whereas Alpern et al. (2019) measured sperm from the female reproductive tract and found three morphs. However, in this study, we also used sperm dissected from the seminal vesicle and found three morphs in all three *pseudoobscura* subgroup species; therefore, it is unlikely to be the sole reason for different results.

All other species were observed to have two sperm morphs, including the basal *subobscura* species subgroup, suggesting that this is the ancestral condition and the production of two parasperm morphs in the *pseudoobscura* subgroup is a derived trait. In future work, it would be of interest to investigate *pseudoobscura* subgroup-specific genes or gene duplications for their potential contribution to the development of multiple parasperm morphs, for example by single cell RNA sequencing (Wei et al., 2024).

Does *D. bifasciata* produce two eusperm morphs?

Cluster analysis raised the possibility of multiple eusperm clusters in *D. bifasciata*, although this clustering was inconsistent between hierarchical clustering and Gaussian mixture modelling. Multiple eusperm clusters may have been identified because of the large range of eusperm lengths observed in *D. bifasciata*. Other than length, no obvious morphological differences were apparent. To establish whether eusperm of all lengths are capable of fertilisation, future work should measure sperm within fertilised eggs (Snook et al., 1994; Snook & Karr, 1998). Electron microscopy would indicate whether ultrastructural morphological differences are present between eusperm of differing lengths.

Function of non-fertilising sperm in sperm heteromorphic *Drosophila*

Although we have not investigated function in this work, it is likely that the morphs have the same or very similar function to those described in *D. pseudoobscura*: fertilising eusperm and non-fertilising protective parasperm (Holman & Snook, 2008). There is evidence that parasperm 2 also has a role in sperm competition in *D. pseudoobscura* (Alpern et al., 2019), and this may also be the case in *D. persimilis* and *D. miranda*, which also produce two parasperm morphs.

As all other *obscura* group species produce a single parasperm morph, it would be of interest to examine whether the single morph has both the protective and competitive functions, or just a single

role. This would indicate whether the *pseudoobscura* subgroup, by evolving a second parasperm, has separated the two parasperm functions into specialised sperm morphs.

Cluster analysis as a method to identify sperm morphs

Previous studies have used kurtosis and ANOVA statistical methods to establish the number of sperm morphs produced by the *obscura* group species. Here we have used a different approach – cluster analysis – to predict the number of morphs produced, based on total sperm length and nucleus length data. We used hierarchical cluster analysis, with group number indicated by gap statistic, and Gaussian mixture modelling. Gap statistic guided hierarchical clustering was more conservative than Gaussian mixture modelling. For most species, the two models indicated different numbers of clusters. We therefore considered morphological differences between clusters, in addition to the statistical approach. Future studies could aim to identify molecular markers of each morph, which would enable easier morph identification in addition to validation of statistical models.

CONCLUSIONS

Species of the *obscura* group produce multiple sperm morphs, a long eusperm morph, which is required for fertilisation, and one or two short parasperm morphs, which protect the eusperm in the female reproductive tract and may also function in sperm competition.

We have confirmed the presence of two parasperm morphs in *D. pseudoobscura* and have identified for the first time that its sister species, *D. persimilis*, and another closely related species, *D. miranda*, also produce two parasperm morphs.

AUTHOR CONTRIBUTIONS

Fiona Messer: Conceptualization; investigation; writing – original draft; methodology; visualization; writing – review and editing; formal analysis. **Helen White-Cooper:** Conceptualization; investigation; funding acquisition; writing – review and editing; project administration; supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data is available at (raw data, R code, model outputs) Cardiff University Research Data (Messer & White-Cooper, 2025) Repository DOI: https://doi.org/10.17035/cardiff.29986768.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Data S1: S1. Raw Data.

S2. R scripts for HCA and GMM modelling.

S3. Model Outputs.

S4. Shapiro–Wilk Normality Test.

S5. Parasperm length as a proportion of total sperm length.

S6. Individual variation in sperm length– Sperm length by male scatterplots.

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